의학박사 학위논문

Helicobacter pylori is associated with *miR-133a* expression through promoter methylation in gastric carcinogenesis

헬리코박터 파일로리가 위암 발병에서 프로모터 메틸화를 통해 *miR-133a* 발현에 미치는 영향

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ABSTRACT

Background/Aims: To investigate whether *Helicobacter pylori* eradication can reverse epigenetic silencing of miRNAs which are associated with *H. pylori*-induced gastric carcinogenesis.

Methods: We examined expression and promoter methylation of *miR-34b/c*, *miR-133a*, *let-7a*, and *let-7i* in gastric cancer cell line, before/after demethylation. Among them, epigenetically controlled miRNAs were identified. Their expression and promoter methylation was examined in human tissues of *H. pylori*-positive gastric cancer(T), *H. pylori*-positive gastritis(H), and *H. pylori*-negative controls(C). We also compared changes of miRNA expression and promoter methylation in *H. pylori*-positive patients who were endoscopically treated for early gastric cancer, between baseline and 1 year later according to eradication status.

Results: In gastric cancer cell line, *miR-34b/c*, and *miR-133a* showed epigenetic silencing. In human tissues, *miR-34b/c* and *miR-133a* showed serial increase of promoter methylation in order of C, H, and T (all, $p<0.01$), and the *miR-133a* expression showed serial decrease (C vs. H, $p=.02$; H vs. T,

0.01; C vs T; p<0.01) while *miR-34b* and *miR-34c* expressions did not. *H. pylori* eradication induced decrease of methylation (p<0.01) and increase of *miR-133a* expression (p=0.03), compared with non-eradication group.

Conclusions: This result suggests *H. pylori* eradication could reverse methylation-silencing of *miR-133a* which is involved in *H. pylori*-induced gastric carcinogenesis.

Keywords: *Helicobacter pylori*; methylation; microRNA; gastric cancer

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LIST OF ABBREVIATIONS

miRNA, microRNA

PCR, polymerase chain reaction

PMR, Percentage of Methylated Reference

IQR, interquartile range

INTRODUCTION

MicroRNAs (miRNAs) are short non-coding RNAs consisting of about 22 nucleotides, which are known to function in post-transcriptional modulation in the way of epigenetic changes such as translational repression or messenger RNA cleavage [1]. Recently, many studies have revealed that various miRNAs are involved in human carcinogenesis. For example, *miR-21* has been shown to be associated with adenoma-carcinoma sequence of colon cancer [2]. Also, *miR-21* and *miR-27a* were reported to be related to gastric carcinogenesis [3, 4]. DNA methylation is another well-known epigenetic phenomenon which induces transcriptional regulation, and it is already established that a great deal of tumor suppressor genes are controlled by promoter methylation. In the meanwhile, miRNA expression is also influenced by epigenetic alterations [5]. Several studies have reported that expression of certain miRNAs are epigenetically regulated by promoter CpG island methylation of the miRNA genes in gastric cancer [6-8].

miR-34b/c was previously found to be silenced by promoter CpG island hypermethylation in colorectal cancer [9] and

gastric cancer [7]. Also, a recent study has shown that aberrant methylation of *miR-34b/c* is associated with metachronous gastric cancer [10]. This study also showed that the promoter methylation of *miR-34b/c* is related with *H. pylori* infection. *Let-7* family is an essential developmental regulator, which is one of the first known miRNAs [11]. *Let-7* genes are thought to be related with tumor suppression, as the expression of which is considerably low in many cancers [12]. In a previous study, *let-7* expression was shown to be down-regulated by Cytotoxin-associated gene A of *H. pylori* by histone modification and DNA methylation of its promoter [8]. *miR-133* is another miRNA which was reported to be associated with *H. pylori* infection. A previous study has revealed that *miR-133* was down-regulated by long-term infection with *H. pylori* in mice [13]. In recent studies, *miR-133* was demonstrated to play tumor suppressive role in gastric carcinogenesis [14-17]. However, whether *H. pylori* eradication therapy could reverse the modulation of these miRNAs has not been clarified yet. Therefore, we aimed to clarify whether the miRNAs which are associated with gastric carcinogenesis could be reversed by *H. pylori* eradication

therapy.

MATERIALS AND METHODS

miRNA and promoter CpG island selection

Based on literature review, *miR-34b/c, let-7,* and *miR-133a* were selected as miRNAs which are possibly associated with *H. pylori*-induced gastric carcinogenesis. *miR-34b* and *miR-34c* are a pair of miRNAs, whose genes are closely located within 418 bp distance in 11q23.1, and they are encoded by common promoter sequence. Therefore we measured expression levels of *miR-34b* and *miR-34c* each, and promoter methylation levels of *miR-34b/c.* Among *let-7* series, *let-7a-3* located in 22q13.31 and *let-7i* in 12q14.1 were selected, as they were the only miRNAs whose promoters contain CpG islands. *miR-133a* is a miRNA encoded by two genes, *miR-133a-1* and *miR-133a-2.* Among them, we measured promoter methylation of *miR-133a-2* gene for the same reason as in *let-7.*

Cell line and demethylation

Gastric cancer cell lines AGS and KATO III were obtained from Korean Cell Line Bank and cultured in RPMI 1640 with L-

glutamine (300mg/L), 25mM HEPES and 25mM NaHCO3, 90%; heat inactivated fetal bovine serum (FBS), 10%. Passage numbers of AGS and KATO III used in the experiment were p69 and p362, each. On day 0, cells were seeded, and the media was added with 2 μM 5-Aza-2'-deoxycitidine (Sigma-Aldrich and Merck KGaA, Darmstadt, Germany), the demethylating agent, on the next day. Cells were treated with 5-Aza-2'-deoxycitidine for 72 hours, while daily replacing the demethylating agent and medium. On day 4, cells were harvested.

Tissue samples

Gastric mucosal tissue samples were obtained from cancerous mucosa of 24 patients with gastric cancer (T, tumor group), antral mucosa of 24 patients with *H. pylori*-positive gastritis (H, *H. pylori*-gastritis group), and antral mucosa of 24 *H. pylori*-negative healthy volunteers (C, control group). All the T, H, and C groups were enrolled between October 2013 and September 2014. *H. pylori* status was considered positive when either the rapid urease test or histologic examination showed a positive result. To assess the effect of *H. pylori*

eradication, non-cancerous mucosal tissues of 24 *H. pylori*-positive early gastric cancer patients who were endoscopically treated were also obtained between January 2012 and December 2012. Then they were randomly assigned to *H. pylori* eradication group (E) and non-eradication group (NE). E group received 20 mg of omeprazole, 1 g of amoxicillin, and 500 mg of clarithromycin, twice a day for 7 days. Twelve months later, gastric antral mucosal tissues were taken from the E and NE groups during the follow-up endoscopy and rapid urease tests were performed as well. Final *H. pylori* status was also evaluated with rapid urease test and histologic examination. When either of those studies showed positive, it was considered as positive. From all tissue samples, degrees of atrophic gastritis and intestinal metaplasia were measured by the Updated Sydney System [18]. And then the remaining tissue samples were restored at −80℃. Also, degrees of neutrophilic infiltration and monocytic infiltration were measured. All the patients enrolled in this study were ≥18 years old, had no other cancer, and were not taking nonsteroidal anti-inflammatory drugs nor proton pump inhibitors. This study was approved by Seoul National

University Hospital Institutional Review Board and complied with the Declaration of Helsinki. From all the participants, informed consent was obtained before tissue retrieval.

Real-time reverse transcription polymerase chain reaction (PCR) of miRNA

The miRNAs were isolated from tissues and cells stored at −80℃ using mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA). Reverse transcription of the miRNAs into the single-stranded cDNAs were performed using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). Quantitative PCR was conducted using TaqMan Universal Master Mix II (Applied Biosystems, Darmstadt, Germany). The relative expression levels of the miRNAs were calculated using the Relative Quantification ($2^{-\Delta\Delta Ct}$) method [19] with duplicate measurements for tissue samples. However, cell line experiments were performed in triplicate to minimize potential errors induced by misidentification or contamination.

DNA isolation and bisulfite modification

DNA was isolated from the tissues and cells using phenol-chloroform extraction method. Bisulfite modification which converts unmethylated cytosine into uracil was performed using EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) (Table 1).

Methylation analysis

For methylation analysis, we used methyLight technique based on quantitative PCR method [20-22] with duplicate measurements for tissue samples and triplicate for cell lines. Pairs of primers and probes to bind bisulfite-converted DNA were designed using the software, Beacon Designer (Premier Biosoft, Palo Alto, CA, USA). To report the levels of DNA methylation, Percentage of Methylated Reference (PMR) was calculated as follows: PMR = 100 x (methylated reaction/ALU)$_{sample}$ / (methylated reaction/ALU)$_{M.SssI}$.

Statistical analysis

To analyze continuous variables with normal distribution, t-test or ANOVA was applied between two or more than two groups, respectively. To analyze continuous variables which are

not normally distributed, Wilcoxon's rank sum test and Wilcoxon's signed rank test were applied for independent and paired samples, respectively. For nominal variables, Chi-square test or Fisher's exact test was applied. When >20% of expected frequencies were ≤5, Fisher's exact test was used. Otherwise, Chi-square test was used. For repeated measures, generalized linear mixed model was applied. *P*-values less than .05 were considered significant. *P*-values were presented without multiple testing. All statistical analyses were performed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA).

Table 1. Primers and probes used in MethyLight assay

Gene	Primer/Probe	Sequence (5'→3')	Length (bp)	T_m (℃)
miR-34b/c	Forward primer	TTCGCGGGGTTTTAAGGACG	20	59
	Reverse primer	CAAACCCTAAAACTAACTCTCTCGAC	26	58.9
	Probe	CCGCCGCTCTAAACGACCGAATAACTAT	28	66.3
let-7a-3	Forward primer	GGGAGTTGAGAGTTAGTATGTCGTT	25	59.2
	Reverse primer	CGCCTCAACCTCCCAAAATACT	22	59.1
	Probe	AACATAAACCACTACGCCCGACCTACTTCC	30	67.7
let-7i	Forward primer	TTTCGAAGGGTGTTGGGGAAC	21	59
	Reverse primer	CCGACGAACATCCCGCAAAA	20	59.6
	Probe	TACCGCCGACTCCGCCAAACAACAA	25	68.1
miR-133a-2	Forward primer	GCTCGATATCTATTCACAACTCACG	25	58.8
	Reverse primer	CGGGGAGGTTATTGCGGTTT	20	58.7
	Probe	CACCACCGTAACGACTACAACGCCAA	26	66.6

RESULTS

Identification of miRNAs silenced by promoter methylation in gastric cancer cell line

In gastric cancer cell lines, AGS and KATO III, the levels of expression of miRNAs and promoter DNA methylation were measured before and after demethylation with 5-Aza-2'-deoxycitidine. After demethylation, the promoter methylation levels of *miR-34b/c* and *miR-133a-2* were decreased by about 30% in AGS and about 50% in KATO III, and the levels of expression of *miR-34b*, *miR-34c*, and *miR-133a* were increased by about 10-fold in AGS and more than 3-fold in KATO III. On the other hand, the promoter methylation levels of *let-7i* were decreased by less than 10% in AGS and 60% in KATO III, and its expression levels were increased by only about 2-fold in AGS and KATO III after demethylation. The promoter methylation levels of *let-7a* were decreased by about 30% in AGS and 50% in KATO III, and the expression levels of which were only increased by 70% in AGS and 40% in KATO III after demethylation (Fig. 1).

Clinicopathological characteristics of enrolled patients

Overall 96 patients were enrolled in this study. Their clinicopathological characteristics are presented in Table 2. Mean age was the highest in T, then H and C group, respectively ($p < 0.01$). Also, the degrees of atrophic gastritis, intestinal metaplasia, neutrophilic infiltration, and monocytic infiltration showed the same pattern (all, $p < 0.01$). However, there was no significant difference in gender proportion among the three groups ($p = 0.25$). Also, there was no significant difference in clinicopathological characteristics between the eradication and non-eradication group, at the time of enrollment.

Promoter methylation and expression levels of miRNAs according to disease status

In human gastric mucosal tissues, we measured promoter methylation levels of *miR-34b/c* and *miR-133a-2*, which were found to be epigenetically silenced by promoter methylation in

above mentioned gastric cancer cell line experiment. In promoters of both miRNAs, the methylation levels were the highest in T, then H and C group, respectively, and the difference between each pair of groups was statistically significant (all, $p < 0.01$, Fig. 2A and 2B). We then examined the expression levels of *miR-34b*, 34c, and 133a. Among them, only *miR-133a* showed serial decrease of expression level in the order of C, H, and then T group. The difference between every two groups was also significant ($p = 0.02$ in C vs. H; $p = 0.01$ in H vs. T; $p < 0.01$ in C vs. T, Fig. 3C). In the meanwhile, although *miR-34b* and *34c* showed lower levels of expression in T compared with C and H, respectively (both *miR-34b* and *34c*, $p < 0.01$ in T vs. C; $p < 0.01$ in T vs. H), they showed no significant difference between C and H group (*miR-34b*, $p = 0.63$; *miR-34c*, $p = 0.90$) (Fig. 3A and 3B).

Promoter methylation and expression level of *miR-133a* under age adjustment

As the age showed significant difference among the three groups, we further performed adjustment analysis. We performed ANCOVA analysis for age adjustment in promoter

methylation and expression levels of *miR-133a*, which was the only miRNA showing serial increase of methylation and decrease of miRNA expression in the progress of *H. pylori*-related gastric cancer development. In this analysis, the promoter methylation was found to be significantly different among C, H, and T group, even after the age adjustment ($p < 0.01$). Besides, age was not a predictor for the promoter methylation ($p = 0.70$). Also, the *miR-133a* expression level showed significant difference among the three groups under the age adjustment ($p = 0.01$), and age was not associated with the *miR-133a* expression level ($p = 0.71$) (Table 3).

Changes of promoter methylation and expression of *miR-133a* according to *H. pylori* eradication status

Among the E and NE groups which all had current *H. pylori* infection, half eradicated *H. pylori* (E group) and half did not (NE group). In E group, the eradication was successful in every patient. One year later, the promoter methylation level tended to decrease in the E group, while it increased in the NE group

(Fig. 4A). On the other hand, *miR-133a* expression showed increasing tendency in the E group, while it did not show significant change among the NE group (Fig. 4B). The difference in changing patterns of promoter methylation among the E and NE groups was analyzed by using generalized linear mixed model. Fixed effects were time and treatment assignment, while individual patients were taken as random effects. This analysis demonstrated an interaction between time and treatment with statistical significance ($p < 0.01$). Likewise, the difference in changes of *miR-133a* expression according to the eradication was evaluated with the same model, where again the interaction between time and eradication showed statistical significance ($p = 0.03$).

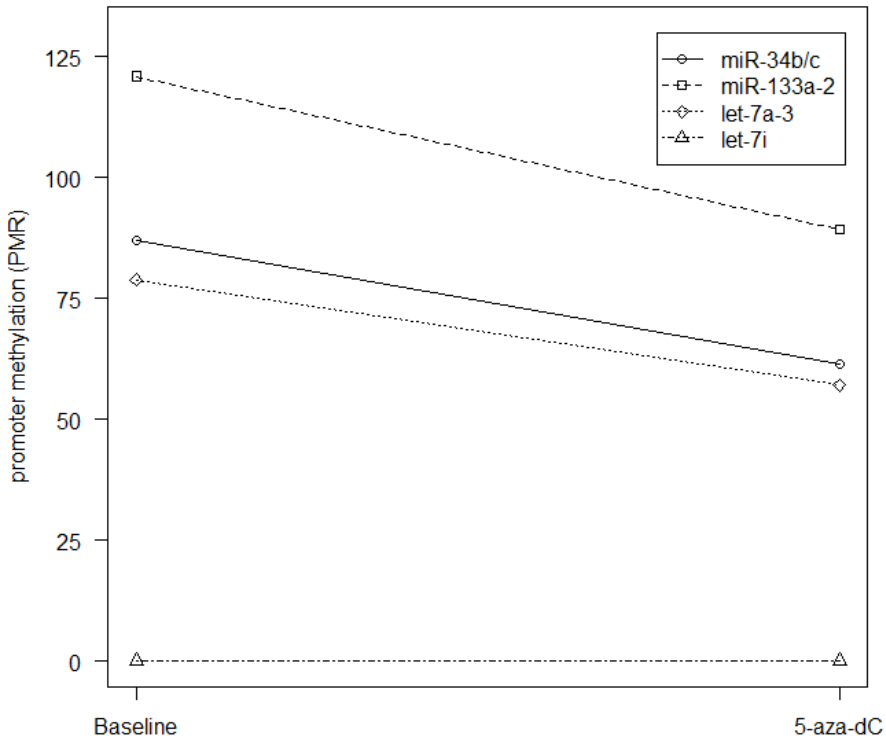

Fig. 1A.

Changes of promoter methylation before and 72 hours after demethylation with 5-Aza-2′-deoxycitidine in gastric cancer cell line AGS.

miRNA, microRNA; PMR, percentage of methylated reference; 5-Aza-dC, 5-Aza-2'-deoxycytidine.

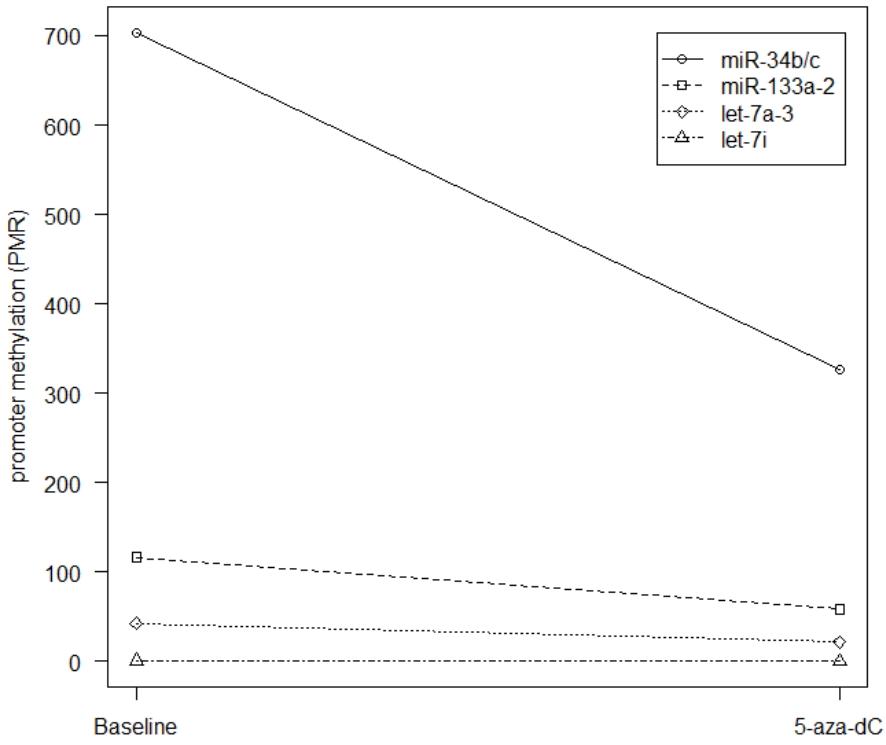

Fig. 1B.

Changes of promoter methylation before and 72 hours after demethylation with 5-Aza-2′-deoxycitidine in gastric cancer cell line KATO III.

miRNA, microRNA; PMR, percentage of methylated reference; 5-Aza-dC, 5-Aza-2'-deoxycytidine.

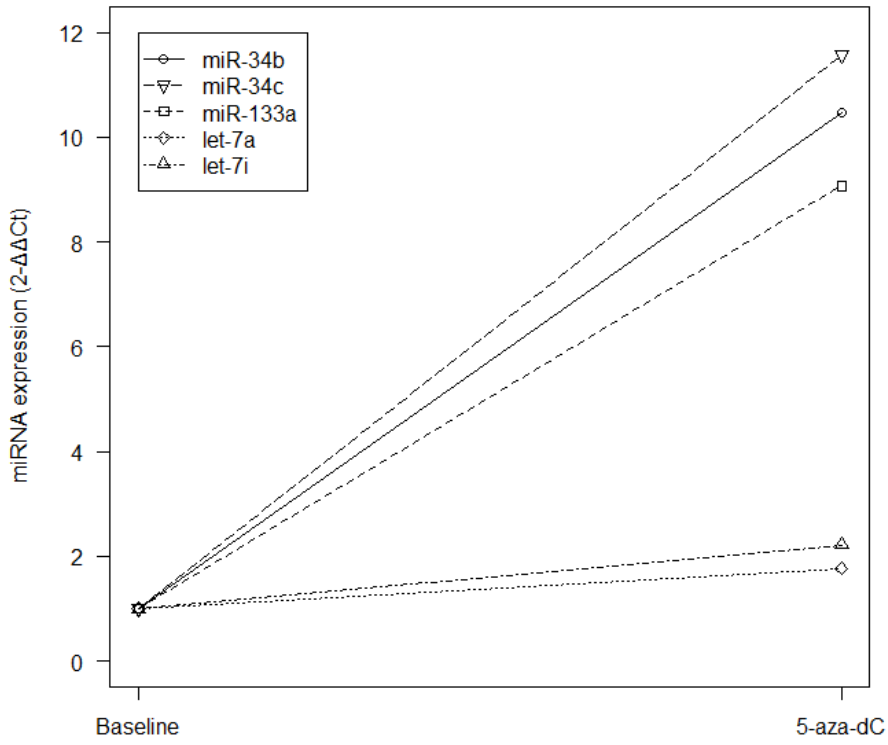

Fig. 1C.

Changes of miRNA expression levels before and 72 hours after demethylation with 5-Aza-2′-deoxycitidine in gastric cancer cell line AGS.

miRNA, microRNA; 5-Aza-dC, 5-Aza-2'-deoxycytidine.

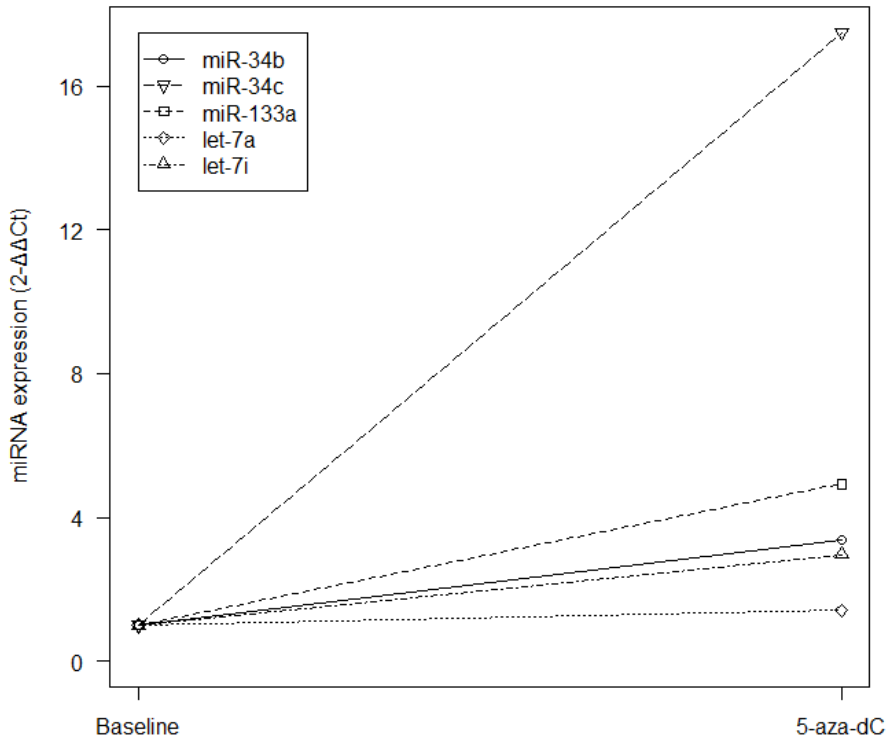

Fig. 1D.

Changes of miRNA expression levels before and 72 hours after demethylation with 5-Aza-2′-deoxycitidine in gastric cancer cell line KATO III.

miRNA, microRNA; 5-Aza-dC, 5-Aza-2'-deoxycytidine.

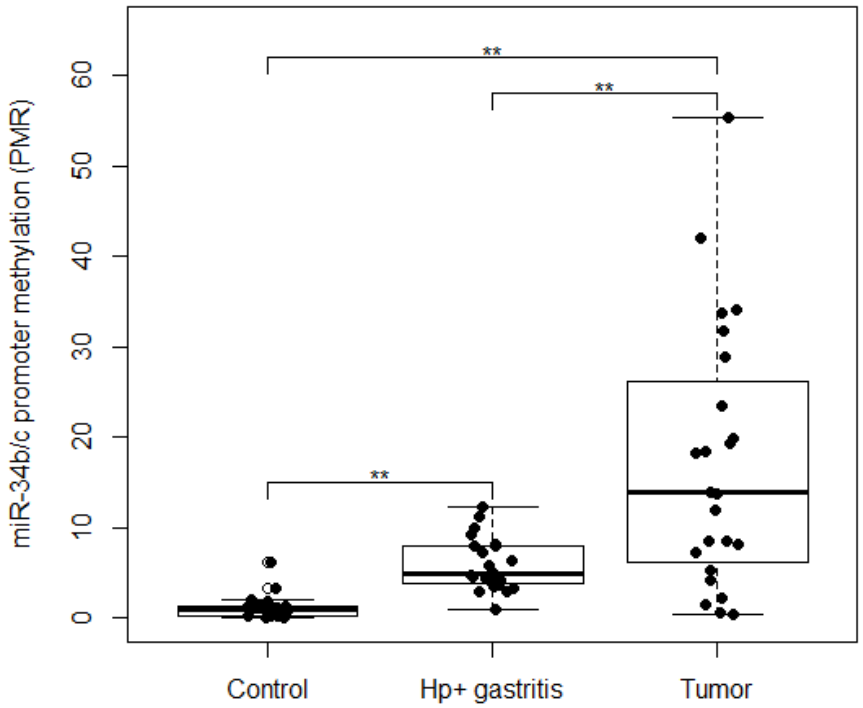

Fig. 2A.

Promoter methylation levels, reported as percentage of methylated reference, of *miR−34b/c* in gastric mucosal tissues of *H. pylori* negative control, *H. pylori* positive gastritis, and *H. pylori* positive gastric cancer.

**, $p < 0.001$

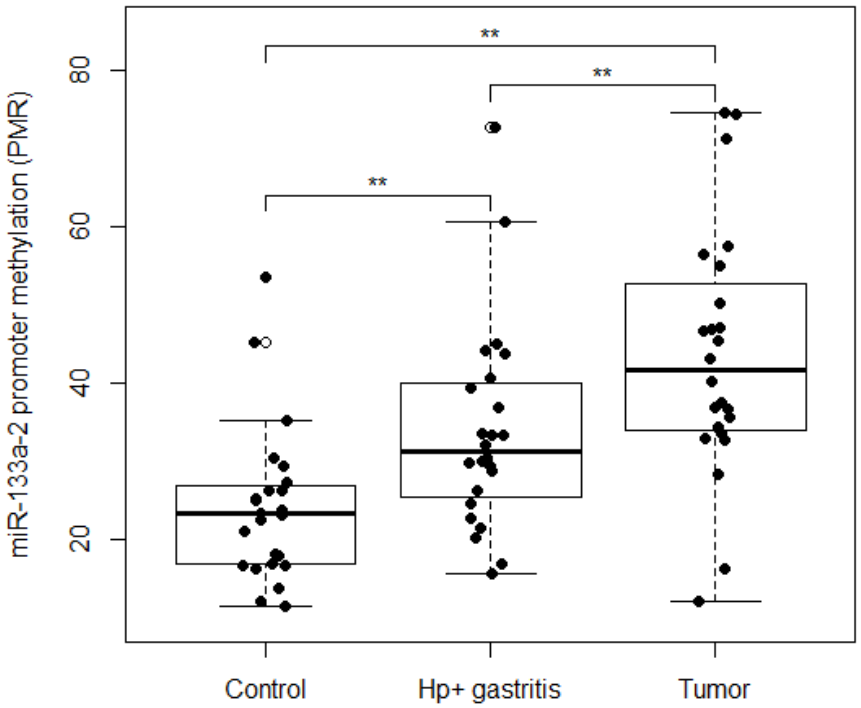

Fig. 2B.

Promoter methylation levels, reported as percentage of methylated reference, of *miR-133a-2* in gastric mucosal tissues of *H. pylori* negative control, *H. pylori* positive gastritis, and *H. pylori* positive gastric cancer.

**, $p < 0.001$

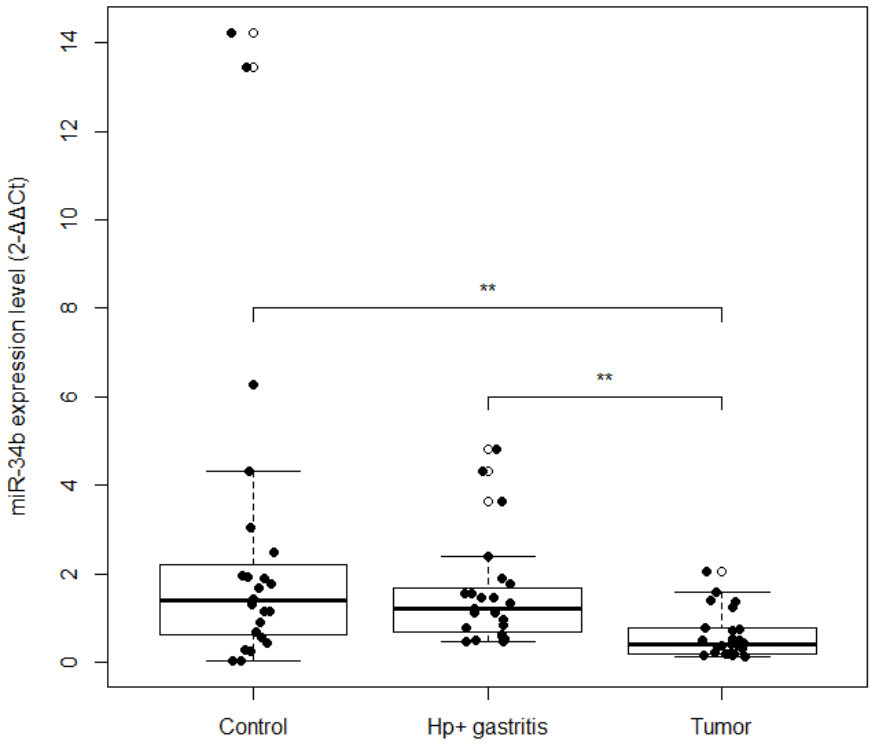

Fig. 3A.

Expression levels of *miR−34b*, reported using the Relative Quantification method ($2^{-\Delta\Delta Ct}$), in gastric mucosal tissues of *H. pylori* negative control, *H. pylori* positive gastritis, and *H. pylori* positive gastric cancer.

**, $p < 0.001$

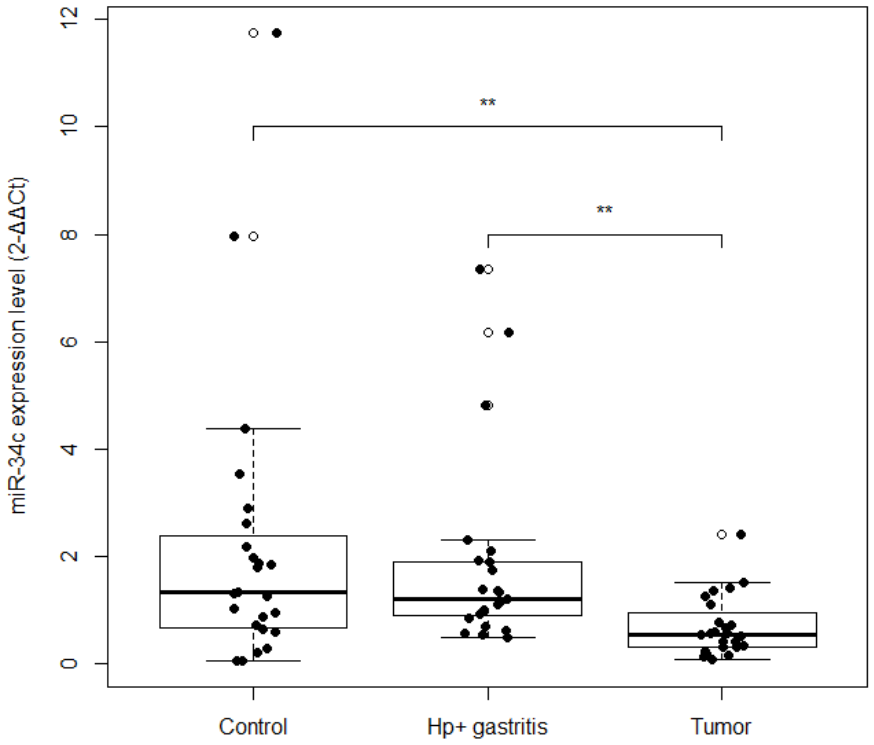

Fig. 3B.

Expression levels of *miR-34c*, reported using the Relative Quantification method ($2^{-\Delta\Delta Ct}$), in gastric mucosal tissues of *H. pylori* negative control, *H. pylori* positive gastritis, and *H. pylori* positive gastric cancer.

**, $p < 0.001$

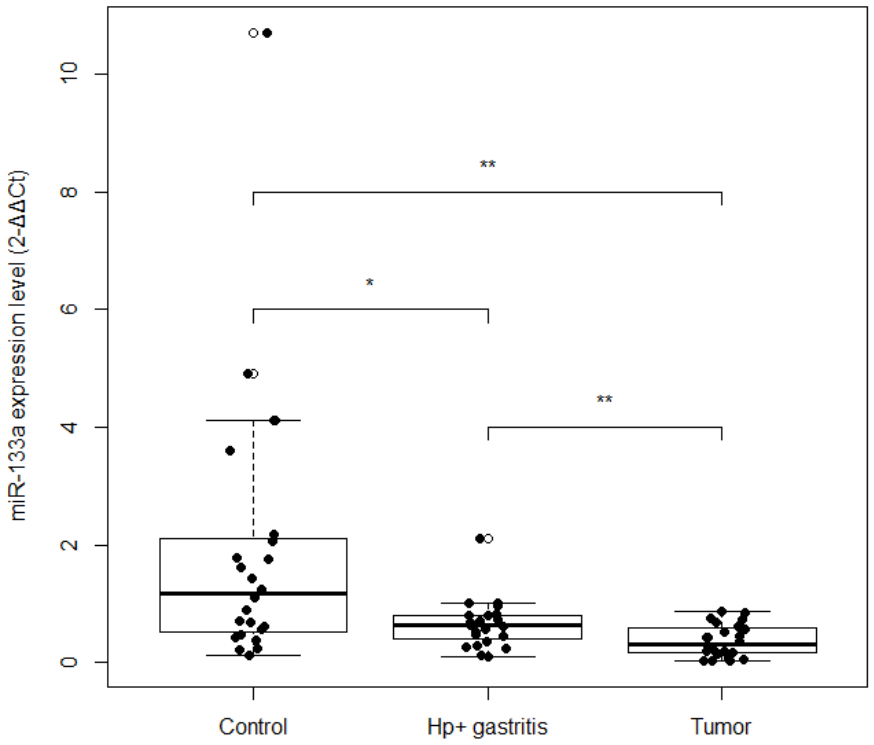

Fig. 3C.

Expression levels of *miR−133a*, reported using the Relative Quantification method ($2^{-\Delta\Delta Ct}$), in gastric mucosal tissues of *H. pylori* negative control, *H. pylori* positive gastritis, and *H. pylori* positive gastric cancer.

*, $p < 0.05$; $p < 0.001$

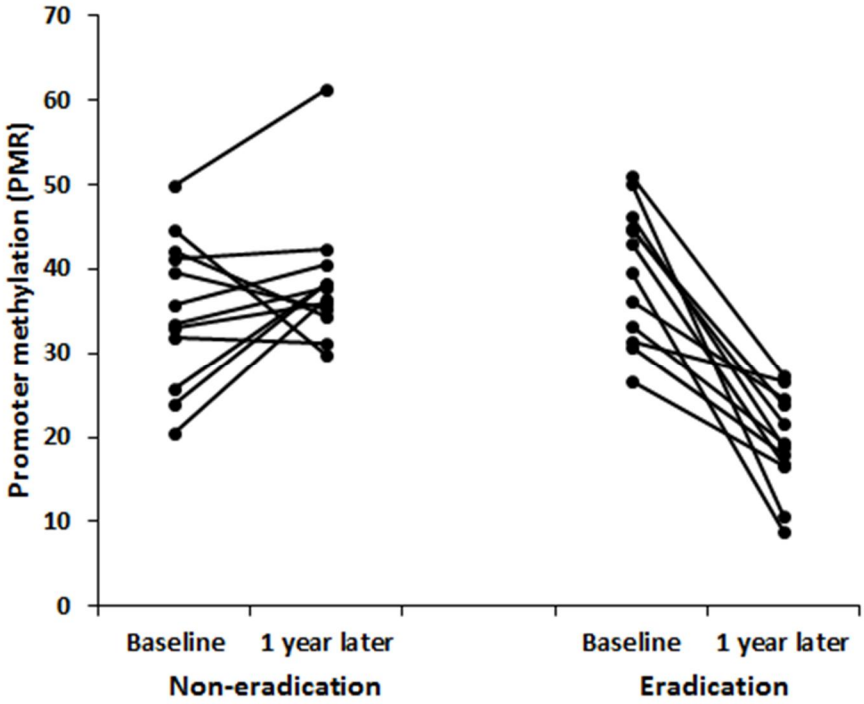

Fig. 4A.

Changes of promoter methylation levels of *miR-133a* before and 1 year after *H. pylori* eradication therapy in normal mucosa of *H. pylori* positive gastric cancer patients.

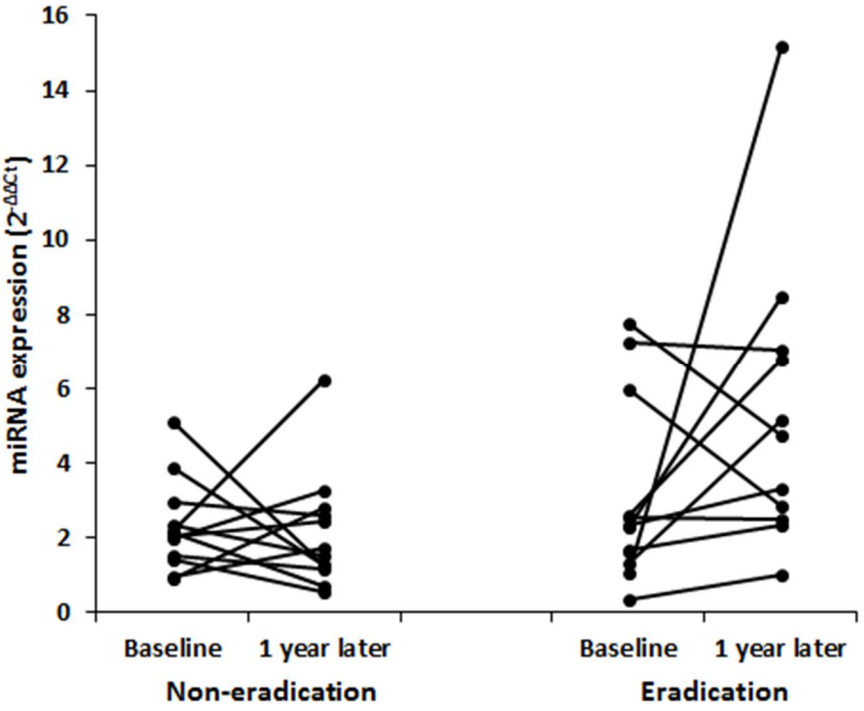

Fig. 4B.

Changes of expression levels of *miR-133a* before and 1 year after *H. pylori* eradication therapy in normal mucosa of *H. pylori* positive gastric cancer patients.

Table 2. Baseline clinicopathological characteristics

		Tumor (n = 24)	*H. pylori*− gastritis (n = 24)	Control (n = 24)	p^*	*H. pylori* eradication (n = 12)	*H. pylori* non−eradication (n = 12)	$p^\dagger$
Age (years), median (IQR)		63.5 (53.5−69.8)	59.0 (42.3−64.0)	42.5 (33.3−52.0)	<0.01	53.5 (50.5−60.8)	57.0 (49.0−67.3)	0.50
Gender, n (%)	Male	14 (58.3)	9 (37.5)	9 (37.5)	0.25	11 (91.7)	8 (66.7)	0.32
	Female	10 (41.7)	15 (62.5)	15 (62.5)		1 (8.3)	4 (33.3)	
Atrophic gastritis, n (%) ‡	Absent	5 (29.4)	9 (39.1)	23 (95.8)	<0.01	2 (16.7)	4 (33.3)	NA
	Mild	3 (17.6)	10 (33.3)	1 (4.2)		5 (41.7)	3 (25.0)	
	Moderate	7 (41.2)	4 (17.4)	0 (0)		5 (41.7)	1 (8.3)	
	Marked	2 (11.8)	0 (0)	0 (0)		0 (0)	1 (8.3)	
Intestinal metaplasia, n (%)	Absent	4 (16.7)	10 (41.7)	24 (100)	<0.01	2 (16.7)	1 (8.3)	0.83
	Mild	7 (29.2)	9 (37.5)	0 (0)		4 (33.3)	5 (41.7)	
	Moderate	7 (29.2)	4 (16.7)	0 (0)		4 (33.3)	4 (33.3)	
	Marked	6 (25.0)	1 (4.2)	0 (0)		2 (16.7)	2 (16.7)	
Neutrophilic infiltration, n (%)	Absent	1 (4.2)	0 (0)	21 (87.5)	<0.01	0 (0)	0 (0)	0.65
	Mild	0 (0)	0 (0)	3 (12.5)		1 (8.3)	0 (0)	
	Moderate	16 (66.7)	18 (75.0)	0 (0)		9 (75.0)	10 (83.3)	
	Marked	7 (29.2)	6 (25.0)	0 (0)		2 (16.7)	2 (16.7)	
Monocytic infiltration, n (%)	Absent	0 (0)	0 (0)	0 (0)	<0.01	0 (0)	0 (0)	1.00
	Mild	2 (8.3)	0 (0)	22 (91.7)		0 (0)	0 (0)	
	Moderate	16 (66.7)	15 (62.5)	2 (8.3)		10 (83.3)	9 (75.0)	
	Marked	6 (25.0)	9 (37.5)	0 (0)		2 (16.7)	3 (25.0)	

IQR, interquartaile range; *H. pylori*, *Helicobacter pylori*

* Comparison was performed among tumor, *H. pylori*−gastritis, and control group.

† Comparison was performed between near tumor eradication and non−eradication group. Results are showing their initial status, not the status after treatment.

‡ Because of missing data, the sum of each does not match the total .

Table 3. ANCOVA analysis for promoter methylation and miR expression levels according to groups under age adjustment.

	Promoter methylation level		miR expression level	
	F	*p*	F	*p*
Age	0.15	0.70	0.14	0.71
Group	7.72	<0.01	5.14	0.01

DISCUSSION

This study demonstrated that suppression of *miR-133a* by its promoter methylation is associated with *H. pylori*-related gastritis and gastric cancer, and it could be reversed by *H. pylori* eradication. As far as we know, this is the first study to verify the reversibility of methylation-induced *miR-133a* down-regulation caused by *H. pylori* infection through *H. pylori* eradication in relation to gastric carcinogenesis.

Although *miR-133a* was previously known as a muscle-specific miRNA, it was recently established to function as a tumor suppressor in various types of cancer, such as bladder cancer [23], head and neck cancer [24], lung squamous cell carcinoma [25], prostate cancer [26], colorectal cancer [27], non-small cell lung cancer [28], cervical cancer [29], pancreatic cancer[30], breast cancer [31], hepatocellular carcinoma [32], and gallbladder cancer [33]. In addition, it has been revealed that *miR-133a* also plays tumor suppressive role in gastric cancer in several studies [14-17]. Its downregulation in gastric cancer was also confirmed in The Cancer Genome Atlas dataset [16]. So far, various target molecules of *miR-133a* have been suggested to be involved in

gastric carcinogenesis and proliferation, such as transcription factor Sp1 [14], IGF1R [15], anti-apoptotic molecules, Mcl-1 and Bcl-xl [16], and ERBB2 [17]. However, the underlying mechanism of *miR-133a* down-regulation has not been well discovered yet. In a previous study, *miR-133a* down-regulation was found to be related with *H. pylori* infection in mice [13]. Herein, we assumed that *miR-133a* would be regulated by promoter methylation induced by *H. pylori* infection. We have demonstrated that epigenetic modification, especially promoter methylation downregulates *miR-133a* by showing the decrease of methylation and increase of *miR-133a* expression by treatment with demethylating agent in gastric cancer cell culture. However, in a previous study, treatment with demethylating agent could not up-regulate *miR-133a* expression in gastric cancer cell line unlike histone methylation inhibitor and deacetylation inhibitor [16]. Although the reason for this discrepancy is unclear, the different time interval before and after the treatment may have influenced. In the previous study, the intervals were 12 hours and 24 hours, whereas ours was 72 hours. Probably, sufficient recovery of *miR-133a* expression by demethylation may take

longer than 24 hours. Similar to our results, there have been a study which revealed silencing of *miR-133a* by DNA methylation, although it was in colorectal cancer [34]. Also another study have shown miR-133a down-regulation by DNA methylation in relation to the development of cardiac hypertrophy induced by air-pollution [35]. However, they showed methylation of only two CpG sites located at the transcription start site of *miR-133a* rather than a CpG island located in the promoter site.

In current study, the promoter methylation showed consistent increase and the *miR-133a* expression showed consistent decrease according to the *H. pylori*-related disease severity: from *H. pylori*-negative control to *H. pylori*-gastritis, and then *H. pylori*-positive gastric cancer. This implies the possibility that the epigenetic silencing of *miR-133a* may be initiated by chronic *H. pylori* infection. As this study only examined *H. pylori*-infected gastric cancer patients, the methylation-silencing of *miR-133a* may not be involved in every gastric carcinogenesis. *H. pylori*-unrelated gastric carcinogenesis may be free from this mechanism. Also, most of the patients had intestinal type gastric cancer except for one diffuse type and

one mixed type cancer in the non-eradication group. Therefore we could not know if it would be the same in diffuse type gastric cancer. Nevertheless, in those with *H. pylori* infection, *miR-133a* down-regulation may play important role in *H. pylori*-related intestinal type gastric cancer development.

Our study result also showed that the down-regulation of *miR-133a* by *H. pylori* infection could be reversed by *H. pylori* eradication, which was also through the decrease of promoter methylation. This finding implies that some epigenetic modification involved in gastric carcinogenesis could be easily treated at certain time after once developed. Moreover, the gastric mucosal tissues used in this eradication experiment were normal-appearing mucosa from those with gastric cancer. Considering the epigenetic field cancerization [36, 37], this result suggests that the reversibility of *miR-133a* expression remains after gastric cancer development. As *miR-133a* down-regulation is already proven to promote tumor proliferation and migration [14-17], treating the modification might be also effective in gastric cancer treatment, not only prevention.

Although there were age differences among the three groups,

the different methylation and *miR-133a* expression levels were the same even after the age adjustment. Also, the small number of patients enrolled in this study is another limitation. Nonetheless, the fact that we showed statistical significance even with this small number further supports our results. Another limitation is that the comparison between cancer and non-cancerous mucosa of cancer patients could not be made in this study because we did not perform paired sampling. This comparison should be made in further researches. Also the fact that this study did not evaluate the mechanism how *H. pylori* modulates methylation or how *miR-133a* regulates tumorigenesis is another limitation. It was over the scheme of this study. We believe this should be further studied in later researches to better understand this phenomenon.

In conclusion, down-regulation of *miR-133a* caused by promoter methylation might be associated with *H. pylori*-related gastric carcinogenesis, and *H. pylori* eradication showed capacity to reverse its expression. Therefore, treating *H. pylori* infection even after gastric cancer development may have some portion of therapeutic role. Also this could be used as a biomarker for early diagnosis and a therapeutic target in gastric

cancer. For such clinical use, further large scale studies are warranted.

REFERENCES

1. Ambros V. microRNAs: tiny regulators with great potential. Cell 2001;107:823-826.
2. Yamamichi N, Shimomura R, Inada K, Sakurai K, Haraguchi T, Ozaki Y, et al. Locked nucleic acid in situ hybridization analysis of *miR-21* expression during colorectal cancer development. Clin Cancer Res 2009;15:4009-4016.
3. Zhang Z, Li Z, Gao C, Chen P, Chen J, Liu W, et al. *miR-21* plays a pivotal role in gastric cancer pathogenesis and progression. Lab Invest 2008;88:1358-1366.
4. Liu T, Tang H, Lang Y, Liu M, Li X. MicroRNA-27a functions as an oncogene in gastric adenocarcinoma by targeting prohibitin. Cancer Lett 2009;273:233-242.
5. Nervi C, Fazi F, Grignani F. Oncoproteins, heterochromatin silencing and microRNAs: a new link for leukemogenesis. Epigenetics 2008;3:1-4.
6. Ando T, Yoshida T, Enomoto S, Asada K, Tatematsu M, Ichinose M, et al. DNA methylation of microRNA genes in gastric mucosae of gastric cancer patients: its possible

involvement in the formation of epigenetic field defect. Int J Cancer 2009;124:2367-2374.

7. Suzuki H, Yamamoto E, Nojima M, Kai M, Yamamono HO, Yoshikawa K, et al. Methylation-associated silencing of microRNA-34b/c in gastric cancer and its involvement in an epigenetic field defect. Carcinogenesis 2010;31;2066-2073.

8. Hayashi Y, Tsujii M, Wang J, Kondo J, Akasaka T, Jin Y, et al. CagA mediates epigenetic regulation to attenuate *let-7* expression in *Helicobacter pylori*-related carcinogenesis. Gut 2013;61:1536-1546.

9. Toyota M, Suzuki H, Sasaki Y, Maruyama R, Imai K, Shinomura Y, et al. Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. Cancer Res 2008;68:4123-4132.

10. Suzuki R, Yamamoto E, Nojima M, Maruyama R, Yamano HO, Yoshikawa K, et al. Aberrant methylation of microRNA-34b/c is a predictive marker of metachronous gastric cancer risk. *J* Gastroenterology 2014;49:1135-1144.

11. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, et al. The 21-nucleotide *let-7* RNA regulates developmental timing in Caenorrhabditis elegans. Nature 2000;403:901-906.

12. Esquela-Kerscher A, Slack FJ. Oncomirs – microRNAs with a role in cancer. Nat Rev Cancer 2006;6:259-269.

13. Saito Y, Suzuki H, Tsugawa H, Suzuki S, Matsuzaki J, Hirata K, et al. Dysfunctional gastric emptying with down-regulation of muscle-specific microRNAs in *Helicobacter pylori*-infected mice. Gastroenterology 2011;140:189-198.

14. Qui T, Zhou X, Wang J, Du Y, Xu J, Huang Z, et al. *MiR-145*, *miR-133a* and *miR-133b* inhibit proliferation, migration, invasion and cell cycle progression via targeting transcription factor Sp1 in gastric cancer. FEBS Lett 2014;588:1168-1177.

15. Gong Y, Fen J, Liu K, Tang LM. Tumor suppressor role of *miR-133a* in gastric cancer by repressing IGF1R. World J Gastroenterol 2015;21:2949-2958.

16. Liu Y, Zhang X, Zhang Y, Hu Z, Yang D, Wang C, et al. Identification of miRNomes in human stomach and gastric

carcinoma reveals *miR-133b/c-3p* as therapeutic target for gastric cancer. Cancer Lett 2015;369:58-66.

17. Li C, Li X, Gao S, Li C, Ma L. microRNA-133a inhibits proliferation of gastric cancer cells by downregulating ERBB2 expression. Oncol Res 2017 doi: 10.3727/096504017X14847395834985. [Epub ahead of print].

18. Dixon MF, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. Am J Surg Pathol 1996;20:1161-1181.

19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402-8.

20. Park SY, Yoo EJ, Cho NY, Kim N, Kang GH. Comparison of CpG island hypermethylation and repetitive DNA hypomethylation in premalignant stages of gastric cancer, stratified for *Helicobacter pylori* infection J Pathol 2009;219:410-416.

21. Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C,

Shibata D, et al. MethyLight: a high-throughput assay to measure DNA methyation. Nucleic Acids Res 2000;28:E32.

22. Ogino S, Kawasaki T, Brahmandam M, Cantor M, Kirkner GJ, Spiegel man D, et al. Precision and performance characteristics of bisulfite conversion and real-time PCR (MethyLight) for quantitative DNA methylation analysis. J Mol Diagn 2006;8:209-17.

23. Chiyomaru T, Enokida H, Tatarano S, Kawahara K, Uchida Y, Nishiyama K, et al. *miR-145* and *miR-133a* function as tumor suppressors and directly regulate FSCN1 expression in bladder cancer. Br J Cancer 2010;102:883-91.

24. Kinoshita T, nohata N, Fuse M, Hanazawa T, Kikkawa N, Fujinura L, et al. Tumor suppressive microRNA-133a regulates novel targets: moesin contributes to cancer cell proliferation and invasion in head and neck squamous cell carcinoma. Biochem Biophys Res Commun 2012;418:378-83.

25. Moriya Y, Nohata N, Kinoshita T, Mutallip M, Okamoto Y, Yoshida S, et al. Tumor suppressive microRNA-133a

regulates novel molecular networks in lung squamous cell carcinoma. J Hum Genet 2012;57:38-45.

26. Kojima S, Chiyomaru T, Kawakami K, Langley RE, Chia WK, van Herk-Sukel MP, et al. Tumor suppressors *miR-1* and *miR-133a* target the oncogenic functionof purine nucleoside phosphorylase (PNP) in prostate cancer. Br J Cancer 2012;106:405-13.

27. Dong Y, Zhao J, Wu CW, Zhang L, Liu X, Kang W, et al. Tumor suppressor functions of *miR-133a* in colorectal cancer. Mol Cancer Res 2013;11:1051-60.

28. Wang LK, Hsiao TH, Hong TM, Chen HY, Kao SH, Wang WL, et al. MicroRNA-133a suppresses multiple oncogenic membrane receptors and cell invasion in non-small cell lung carcinoma. PLoS One 2014;9:e96765.

29. Song X, Shi B, Huang K, Zhang W. *miR-133a* inhibits cervical cancer growth by targeting EGFR. Oncol Rep 2015;34:1573-80.

30. Qin Y, Dang X, Li W, Ma Q. *miR-133a* functions as a tumor suppressor and directly targets FSCN1 in pancreatic cancer. Oncol Res 2013;21:353-63.

31. Cui W, Zhang S, Shan C, Zhou L, Zhou Z. microRNA-

133a regulates the cell cycle and proliferation of breast cancer cells by targeting epidermal growth factor receptor through the EGFR/Akt signaling pathway. FEBS J 2013;280:3962-74.

32. Zhang W, Liu K, Liu S, Ji B, Wang Y, Liu Y. MicroRNA-133a functions as a tumor suppressor by targeting IGF-1R in hepatocellular carcinoma. Tumour Biol 2015;36:9779-88.

33. Huang Y, Wu Y, Dong J, Han D, Yang S, Jiang L. MicroRNA-133a-3p exerts inhibitory effects on gallbladder carcinoma via targeting RBPJ. Am J Cancer Res 2016;6:2448-62.

34. Chen WS, Leung CM, Pan HW, Hu LY, Li SC, Ho MR, et al. Silencing of *miR-1-1* and *miR-133a-2* cluster expression by DNA hypermethylation in colorectal cancer. Oncol Rep 2012;28:1069-76.

35. Huang L, Xi Z, Wang C, Zhang Y, Yang Z, Zhang S, et al. Phenathrene exposure induces cardiac hypertrophy via reducing miR-133a expression by DNA methylation. Sci Rep 2016;6:20105.

36. Ramachandran K, Singal R. DNA methylation and field

cancerization. Epigenomics 2012;4:243–5.

37. Ushijima T. Epigenetic field for cancerization: its cause and clinical implications. BMC Proc 2013;7:K22.

국문 초록

배경 및 목적: 헬리코박터 파일로리 제균치료가 헬리코박터 연관 위암 발병과 관련된 마이크로 RNA 의 후성학적 억제를 역전시킬 수 있는지 알아보고자 하였다.

방법: 위암 세포주에서 탈메틸화 전후의 *miR-34b/c*, *miR-133a*, *let-7a* 및 *let-7i* 의 발현정도와 프로모터 메틸화 정도를 측정하여 후성학적으로 조절되는 마이크로 RNA 를 찾아낸 후, 헬리코박터 파일로리 양성 위암 (T), 헬리코박터 파일로리 양성 위염 (H), 및 헬리코박터 파일로리 음성 대조군 (C)의 인체 조직에서 이들 마이크로 RNA 의 발현과 프로모터 메틸화를 측정하였다. 또한 조기위암으로 내시경적 치료를 받은 헬리코박터 파일로리 양성 환자들을 대상으로 제균여부에 따라 기저 상태와 1년 후의 마이크로 RNA 발현 및 프로모터 메틸화의 변화를 비교하였다.

결과: 위암 세포주에서 *miR-34b/c* 와 *miR-133a* 는 후성학적 억제가 확인되었다. 인체 조직에서는 C, H,

T 군의 순서로 *miR-34b/c* 와 *miR-133a* 의 프로모터 메틸화가 증가하였으며 ($p < 0.01$), *miR-133a* 의 발현이 감소하였으나 ($p < 0.01$), *miR-34b* 와 *miR-34c* 의 발현은 그렇지 않았다. 헬리코박터 파일로리 제균치료는 제균하지 않은 군에 비하여 *miR-133a* 의 메틸화를 감소시키고 ($p < 0.01$), 발현을 증가시켰다 ($p = 0.03$).

결론: 헬리코박터 파일로리 제균치료는 헬리코박터 파일로리 연관 위암 발생과 관련이 있는 *miR-133a* 의 메틸화를 통한 발현 억제를 역전시킬 수 있는 것으로 나타났다.

주요어 : 헬리코박터 파일로리 ; 메틸화 ; 마이크로 RNA ; 위암
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